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Isolation of strains from a bio-hydrogen plant: Novel [FeFe]-hydrogenases for Exploitation in Biotechnology

Francesca Valetti^{1*}, Simone Morra¹, Mariaconcetta Arizzi¹, Veronica Sarasso¹, Sheila J. Sadeghi¹, Barbara La Licata², Fabio Sagnelli², Paola Zitella² and Gianfranco Gilardi¹

¹Department of Life Sciences and Systems Biology, University of Torino, Torino, Italy. ²Bioenergy Lab, Enviroment Park, Torino, Italy

*francesca.valetti@unito.it

INTRODUCTION

Hydrogen is considered a promising fuel that can replace fossil fuels in the future. The ability of various microorganisms to produce hydrogen from low cost waste materials is of high interest (Kapdan 2006) and several bioreactors and pilot plants are being tested (La Licata 2011). Nevertheless, very often the microbiological consortium remains uncharacterized. Moreover, there is much interest in the identification of new [FeFe]-hydrogenases, enzymes responsible for H₂ evolution. [FeFe]-hydrogenases can then be used as very efficient catalysts for the development of bio-hybrid devices that produce H₂ (Cracknell 2008).

The main aim of this study is to characterize the culturable bacteria in the sludge from a pilot plant fed with waste materials that produces efficiently high amount of hydrogen. The best hydrogen producers are then selected for gene expression studies. Five new previously uncharacterized [FeFe]-hydrogenases are cloned, recombinantly purified and exploited in the construction of a biotechnological device.

PILOT PLANT

The pilot plant was designed and built to produce biohydrogen from waste materials taking into consideration the environmental and economical feasibility. It was fed with a mixture of fruit and vegetables scraps from unsold stocks. The optimised process granted high percentage of hydrogen with good yield.



Figure 1. Pilot plant.

Average Productivity	0.72 L/L day
Productivity	0.9 L/h
Temperature	Room temp.
HRT	30 h
H ₂ max	54 %
Yield	45 mL H ₂ /g VS
pH	5-6

Table 1. Parameters and yield for optimized dark fermentation.

BACTERIAL CONSORTIUM CHARACTERIZATION

The culturable bacteria were isolated and identified by means of 16S rDNA gene analysis. Each species was tested for its ability to produce hydrogen.

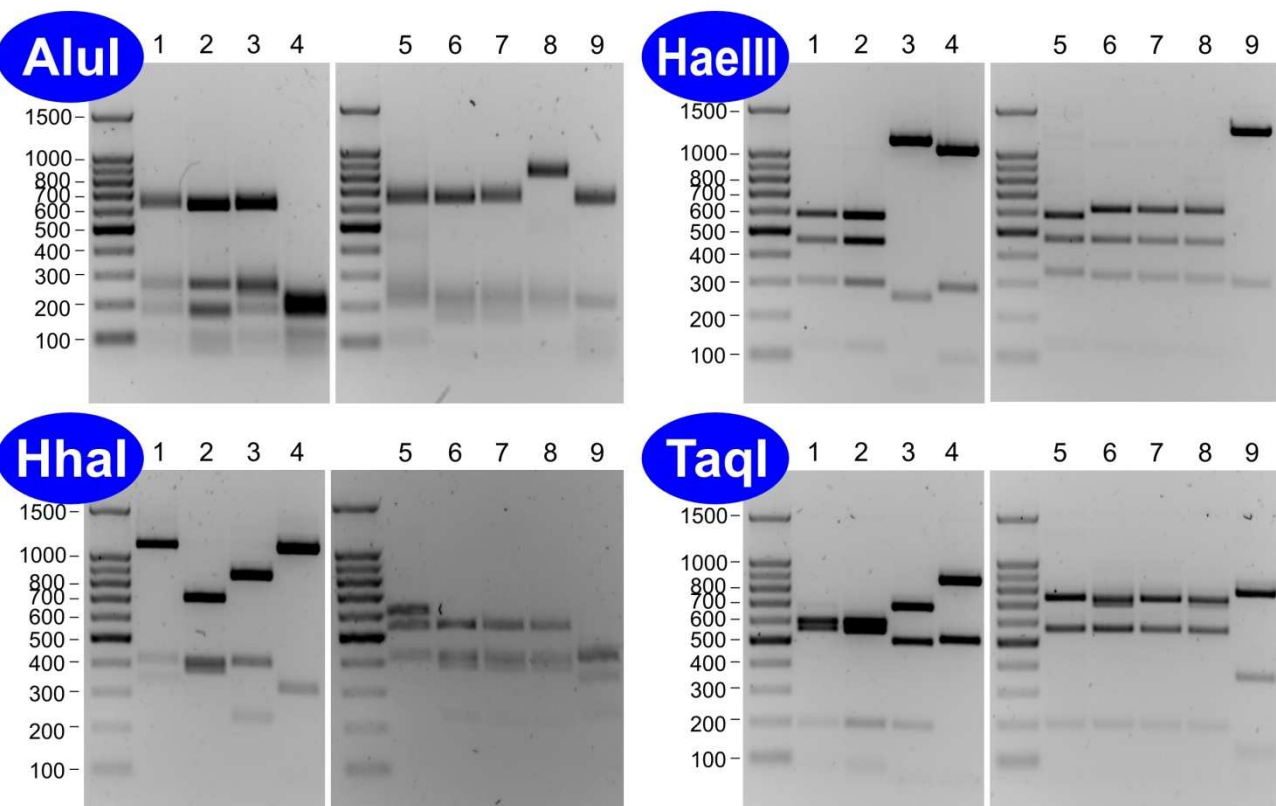


Figure 2. 16S rDNA gene RFLP of the 9 isolated bacteria.

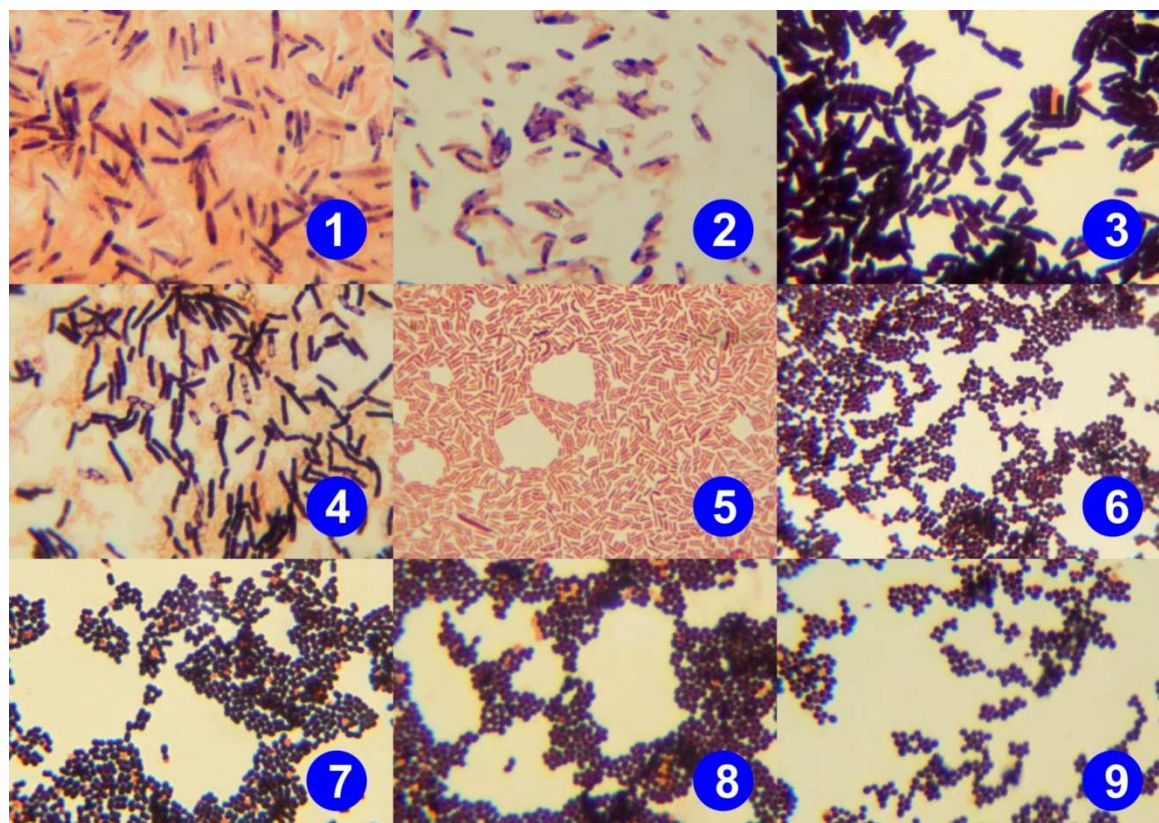
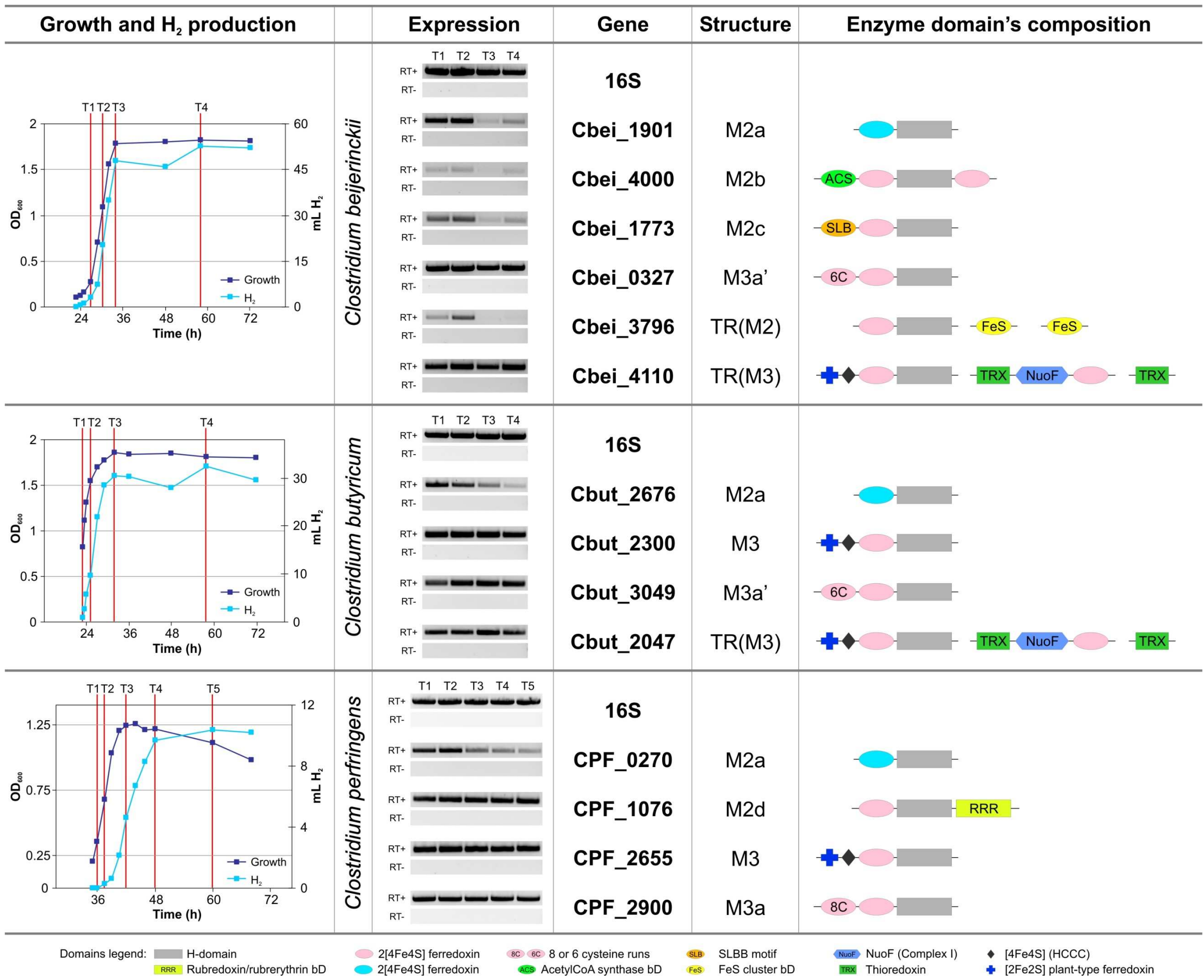


Figure 3. Gram staining of the 9 isolated bacteria.

ID	Species	Morphology	Gram	O ₂	Kan	mL H ₂ /g glucose
1	<i>Clostridium beijerinckii</i>	bacilli	+	-	+	247.2 ± 9.6
2	<i>Clostridium butyricum</i>	bacilli	+	-	+	136.3 ± 4.6
3	<i>Clostridium perfringens</i>	bacilli	+	-	+	130.6 ± 3.0
4	<i>Clostridium bifementas</i>	bacilli	+	-	-	97.1 ± 9.2
5	<i>Lactobacillus plantarum</i>	bacilli	+	+	+	n.d.
6	<i>Enterococcus sp.</i>	cocci	+	-	-	n.d.
7	<i>Enterococcus devriesei</i>	cocci	+	-	-	n.d.
8	<i>Enterococcus sp.</i>	cocci	+	+	+	n.d.
9	<i>Staphylococcus hominis</i>	cocci	+	+	-	n.d.

Table 2. Summary of the 9 bacteria identified in the H₂ producing sludge. n.d. = not detectable.

[FeFe]-HYDROGENASE GENES EXPRESSION STUDY



[FeFe]-HYDROGENASE CLONING AND RECOMBINANT EXPRESSION

Five genes encoding for previously uncharacterized [FeFe]-hydrogenases were cloned into a specific vector suitable for the recombinant expression in *E. coli* (King 2006): Cbei_1773, Cbei_0327, Cbei_1901, Cbei_4110 (from *C. beijerinckii*) and CPF_2655 (from *C. perfringens*).

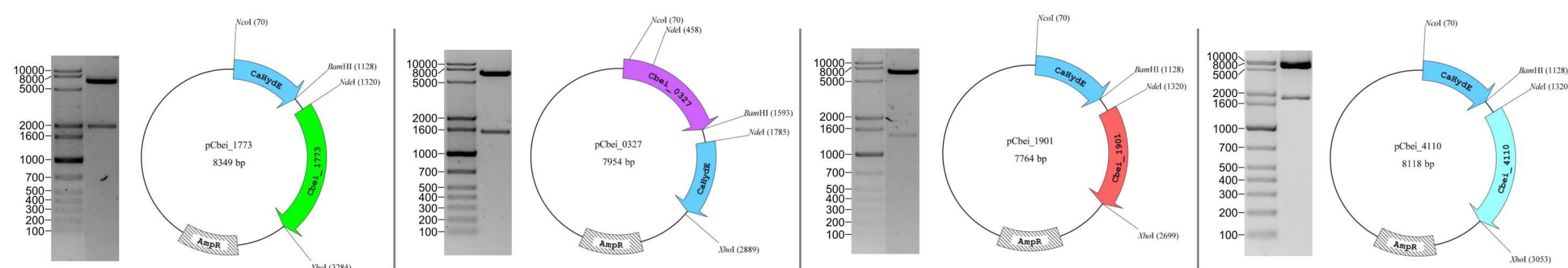


Figure 4. Expression vectors with the five cloned genes.

Three of these genes could be readily expressed in the active form and two of them have been purified by affinity chromatography under strict anaerobic conditions. The two purified proteins catalyzed H₂ evolution at a rate of: CPF_2655 and Cbei_1773.

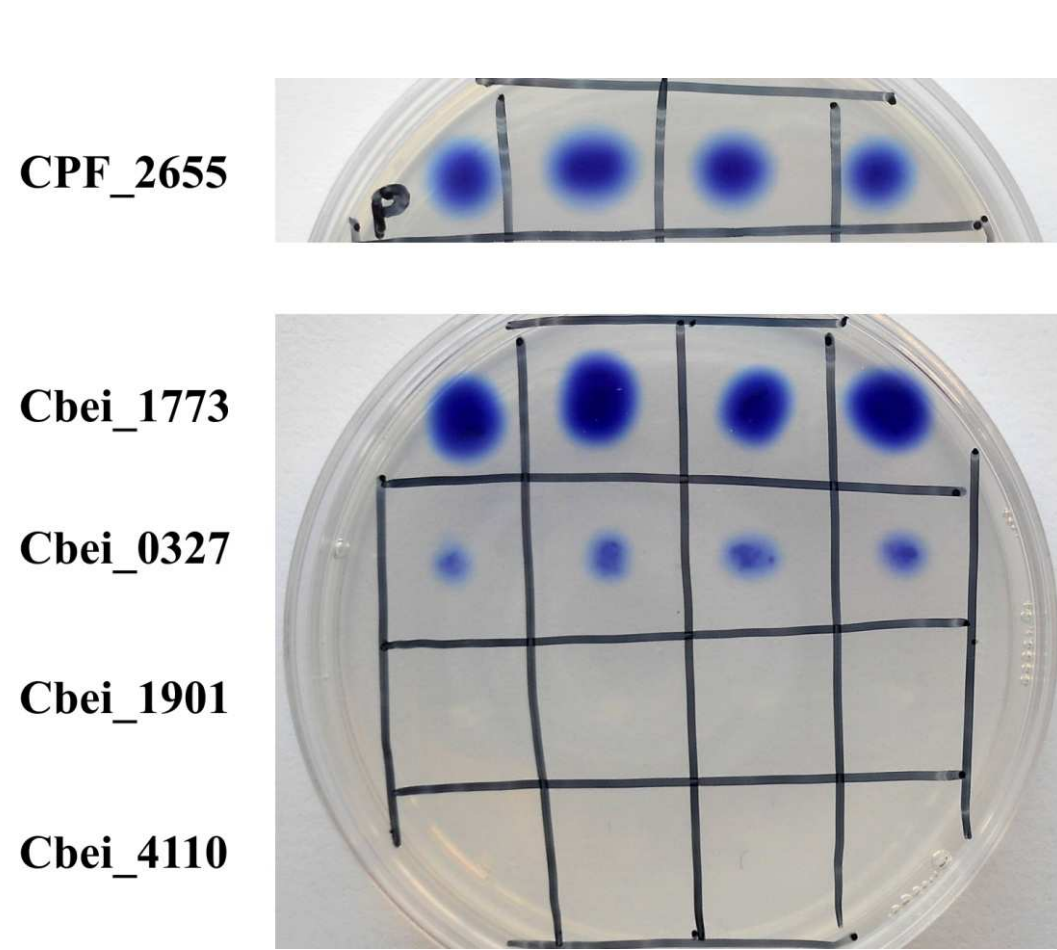


Figure 5. Activity test of the five cloned genes in the *E. coli* recombinant expression system.

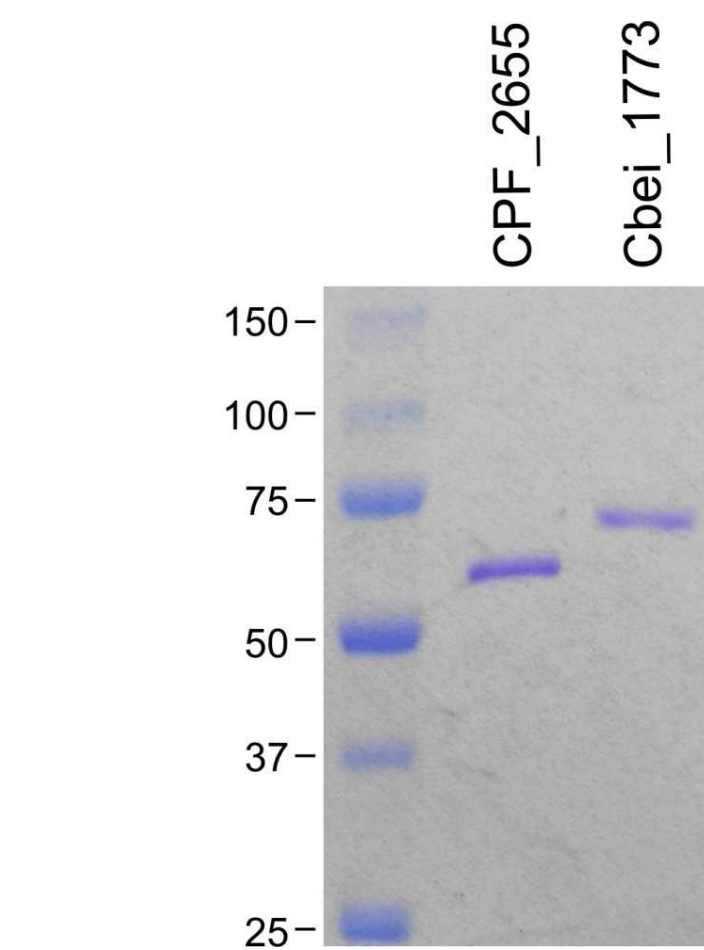


Figure 6. Coomassie stained SDS-PAGE of the two purified enzymes: CPF_2655 (64.7 kDa) and Cbei_1773 (73.1 kDa).

EXPLOITATION: ENZYME IMMOBILIZATION ON TiO₂ ELECTRODES

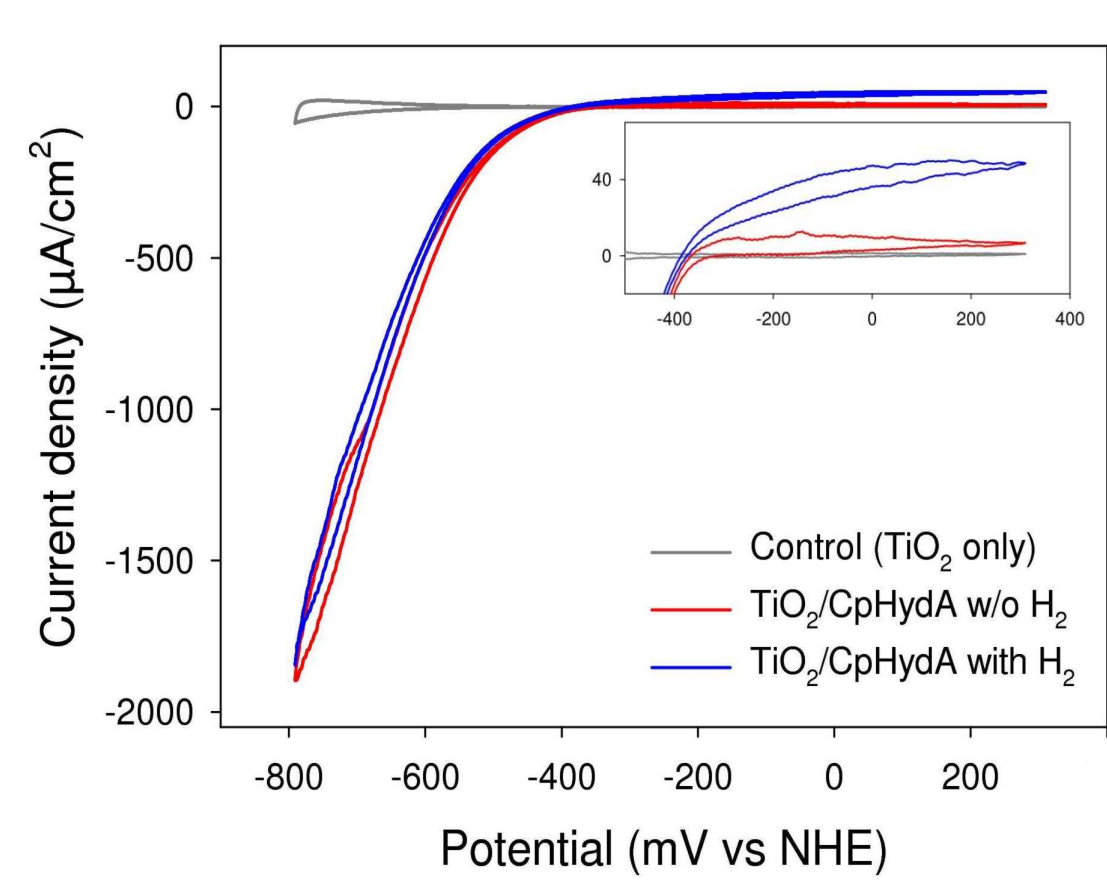


Figure 7. Cyclic voltammetry of CpHydA on TiO₂ electrodes. Voltammograms were acquired under nitrogen atmosphere in the presence or in the absence of H₂ to observe both reductive and oxidative catalytic currents (inserts).

CpHydA was immobilized by adsorption on TiO₂ electrodes. Cyclic voltammetry was used to demonstrate CpHydA immobilization, with specific electron transfer; in particular, high reductive catalytic currents due to H⁺ reduction into H₂ were observed.

Hydrogen evolution was demonstrated applying a fixed potential (-741 mV vs NHE) to the electrode for 2 hours. Analysis of the gas phase by gas chromatography showed H₂ production with very high efficiency: 98±1 %.

CONCLUSIONS AND FUTURE PERSPECTIVES

The sludge from a pilot plant producing hydrogen in high percentage (up to 54%) from waste materials was found to contain a consortium of 9 different bacterial strains, 4 of which were hydrogen producing *Clostridia*. The expression of all the [FeFe]-hydrogenases of *C. beijerinckii*, *C. butyricum* and *C. perfringens* was studied by RT-PCR during the growth. Five previously uncharacterized [FeFe]-hydrogenases were cloned in a vector for the recombinant expression in *E. coli*. CPF_2655 and Cbei_1773 were affinity purified and CPF_2655 was exploited to develop a bio-inspired biotechnological cathode able to produce H₂ with extremely high efficiency. In the future all the five enzymes will be purified and characterized to test their potential as *in vitro* H₂ producing catalysts.

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